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β-Carotene Impacts the Liver microRNA Profile in a Sex-Specific Manner in Mouse Offspring of Western Diet-Fed Mothers: Results from Microarray Analysis by Direct Hybridization

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Abstract: Maternal unbalanced diets cause adverse metabolic programming and affect the offspring's liver microRNA profile. The liver is a site of β-carotene (BC) metabolism and a target of BC action. We studied the interaction of maternal Western diet (WD) and early-life BC supplementation on the epigenetic remodeling of offspring's liver microRNAs. Mouse offspring of WD-fed mothers were given daily a placebo (controls) or BC during suckling. The liver miRNAome was analyzed in recently weaned animals by microarray hybridization. BC impacted the liver miRNAome differently in male and female offspring, with no overlap in differentially expressed (DE) miRNAs between sexes and more impact in females. Bioinformatic analysis of DE miRNA predicted target genes revealed enrichment in biological processes/pathways related to metabolic processes, regulation of developmental growth and circadian rhythm, liver homeostasis and metabolism, insulin resistance, and neurodegeneration, among others, with differences between sexes. Fifty five percent of the overlapping target genes in both sexes identified were targeted by DE miRNAs changed in opposite directions in males and females. The results identify sex-dependent responses of the liver miRNA expression profile to BC supplementation during suckling and may sustain further investigations regarding the long-term impact of early postnatal life BC supplementation on top of an unbalanced maternal diet.

Keywords: carotenoids; β-carotene; liver; microRNAs; western diet; lactation; weaning; early-life programming

1. Introduction

Carotenoids are natural pigments produced by vegetables, algae, and photosynthetic bacteria that play an important role in human health [1]. Among the carotenoids, β-carotene (BC) has been widely studied for its role as a precursor of vitamin A. In mammals, vitamin A and BC are crucial for

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the normal function of vision [2], skin [3], energy and lipid metabolism [4], and neurological processes [5,6], among others. Due to its potential to scavenge free radicals and its interaction with specific cellular signaling pathways, BC has been associated with protective effects against oxidative stress- and inflammation-related diseases, including cardiovascular diseases, type 2 diabetes, obesity, and some types of cancer [7], which are often associated with unbalanced obesogenic diets.

The liver is an important organ for vitamin A and BC metabolism, and BC has recognized hepatoprotective effects in adult animals [8,9]. Endogenous microRNAs (miRNAs, miR) contribute to the control of hepatic function and metabolism [10,11]. The miRNAs are short (20–22 nucleotides) non-coding RNA molecules that act at the post-transcriptional level to regulate (generally inhibit) gene expression. Interestingly, the ablation of carotenoid cleavage enzymes (BCO1 and BCO2) induces hepatic steatosis in mice by altering the farsenoid X receptor/miR-34a/sirtuin 1 pathway [12], suggesting a connection between BC metabolism and metabolic health through the regulation of specific miRNAs in the liver.

Studies in human and animal models indicate that maternal high-fat diet feeding and obesity result in adverse metabolic programming of the offspring [13]. miRNAs are epigenetic agents [14] that may play a role in the mechanisms of metabolic programming by dietary and other factors in early life. Besides miRNAs present at variable levels in breast milk [15,16], changes in endogenous miRNAs in offspring tissues could be involved. In particular, animal studies indicate that maternal high-fat diet affects miRNA expression in the liver of the fetus, recently weaned offspring, and adult offspring [17–21]. The metabolic programming activity of BC, in its turn, has been little studied, but emerging evidence suggests that carotenoids in human milk may play important roles in infant nutrition and development [22], and it is known that breast milk BC content is decreased in obese mothers [23]. Supplementation with BC and preformed vitamin A during lactation were shown to have distinct effects on adipose tissue DNA methylation in rats [24]. However, to our knowledge, no studies have addressed the possible interactive effects of a maternal obesogenic diet and BC supplementation during lactation on the miRNA expression profile in the offspring's liver.

In this work, we hypothesized that BC supplementation during lactation could beneficially modify miRNA expression in the liver of offspring exposed to an unbalanced diet during development. To test this hypothesis, mouse offspring born to dams fed an obesogenic Western Diet (WD) rich in fat and sucrose were orally supplemented with placebo (controls) or BC during the suckling period. The liver miRNA profile was analyzed in the recently weaned animals for differential expression using a microarray direct hybridization method. To elucidate possible post-transcriptional regulation by these miRNAs, we constructed pathway interaction and miRNA-gene regulatory networks through integrated analysis of the crucial miRNAs showing modulation.

2. Materials and Methods

2.1. Experimental Design

The animal protocol of this study (**Figure 1**) was reviewed and approved by the Animal Experimentation Ethics Committee (CEEA) of the University of the Balearic Islands (#ECC/566/2015 of March 20). Institutional use and care guidelines for laboratory animals were followed. C57BI/6J female mice kept under constant conditions of 12/12-h light-dark cycle at room temperature of 22 °C, and with free access to food and water were used. The female mice were switched from the standard chow diet (LASQCdiet®Rod14-H, Lage, Germany) to a WD pellet diet (D12079B, Research Diets, New Brunswick, NJ, USA) 23 days before mating with males of the same strain fed the standard chow. The dams continued to be fed the WD during the whole gestation and lactation period. From day 2 post-delivery until weaning (on postnatal day 21), the pups were orally treated daily, with the aid of a pipette, with 10 μL olive oil (control group) or the same volume of β-carotene (BC group) dissolved in olive oil. The amount of supplemented BC increased progressively from 10 μg BC per pup per day on postnatal d2 to 18 μg on d20 and corresponded to ~three-fold the vitamin A ingested daily through maternal milk. The daily amount of BC to be administered was calculated from the reported vitamin A concentration as retinyl ester in mouse milk [25], the estimated daily milk intake

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of mouse pups (which increases along the suckling period) [26], and the vitamin A equivalency of BC (VEB) in oil. We used a VEB of 2.5:1, which is based on the reported micrograms of BC in oil required to form 1 μg retinol in children, as in previous work with a similar animal study design [27]. At weaning, offspring were separated by sex, making up four experimental groups: control male and female mice (C-M and C-F, respectively) and BC-supplemented male and female mice (BC-M and BC-F). The animals continued to be fed with WD until sacrifice on postnatal day 26, when livers were collected, frozen immediately in liquid nitrogen, and stored at -80 ˚C until RNA extraction and analysis.

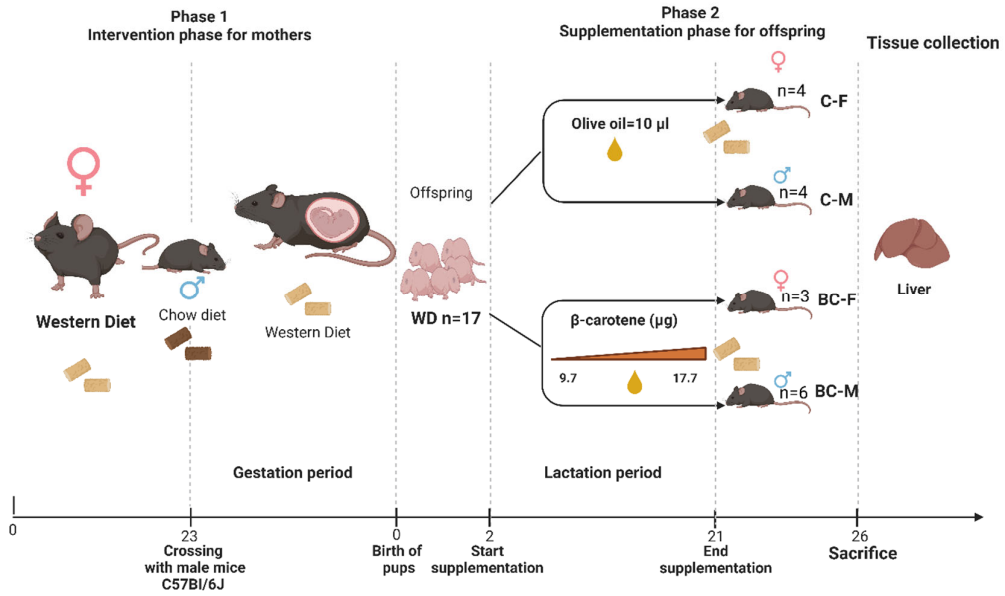

Figure 1. Diagram of the experimental study design. Phase 1, intervention phase for mothers; phase 2, supplementation phase for offspring during the lactation period. The mothers were kept on the WD during the lactation period. WD, Western Diet; C-F, Control-female; C-M, Control-male; BC-F, β-carotene-female; BC-M, β-carotene-male. Created with BioRender.

2.2. RNA Isolation and Quantification

The mirVana™ miRNA Isolation Kit (Ambion by Life Technologies, Grand Island, NY, USA) was used to extract miRNA from liver samples following the manufacturer's instructions. First, 0.024 ± 0.005 g of liver samples were homogenized in 250 μL of lysis binding buffer, on ice. A spike-in of *Arabidopsis thaliana* microRNA (ath-miR-159a, 1 pmol, 2 μL) was added to each sample as a standard, vortexed for 30 sec, and incubated on ice for 5 min. Then, 1/10 volume of miRNA Homogenate Additive (55 μL) was added to the homogenate, mixed by vortexing, and left for 10 min on ice. Subsequently, 250 μL phenol/chloroform was added and vortexed for 1 min. The mixture was then centrifuged for 10 min at 10,000 g at 4°C to separate the aqueous phase from the organic phase. The aqueous phase (upper) was pipetted and transferred to an RNase-free Eppendorf (200 μL) and 1.25 volumes of 100% ethanol were added at room temperature and mixed by inversion (5 times). Each sample was passed through the columns in aliquots of 250 μL and centrifuged for 30 sec at 10,000 g, the eluate was discarded. This was repeated until all the lysate/ethanol mixture was passed through the filter. The filter/column was washed with 700 μL of miRNA wash solution 1 and centrifuged for 10 sec at 10,000 g, and then the column was washed with 500 μL of wash buffer solution 2/3, centrifuged twice at 10,000 g for 10 sec, and the eluate was discarded each time. A spin of 1 min was performed to remove the remains of the filter. The columns were transferred to a new collection RNase-free tube, and 50 μL of RNase-free water at 95°C were added and incubated for 2 min. Finally, it was centrifuged for 30 sec at maximum speed, and the RNA eluate was collected and stored at -80°C. Nucleic acids extracted were quantified by spectrophotometry with NanoDrop ND-1000

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(ThermoFisher Scientific, Waltham, MA, USA) at 260 nm, using 2 µL, which allows it to be quantified in ng/µL with reproducibility and precision. Samples with nucleic acid concentration >100 ng/µL were used. RNA quality and integrity were checked by 260/280 ratio and confirmed by 1% agarose gel electrophoresis.

2.3. miRNA Assessment

150 ng of liver RNA/miRNA was used as input for miRNA profiling using the n-counter flex NanoString Technology. Mature miRNAs underwent an annealing procedure for 13 min with the following conditions: 94°C for 1 min, 65°C for 2 min, and 45°C for 10 min. Then, miRNAs were ligated to a species-specific tag sequence (miRtag) via a thermally controlled splinted ligation at 48°C for 3 min, 47°C for 3 min, 46°C for 3 min, 45°C for 5 min, and 65°C for 10 min (24 min in total). The unligated miRtags were removed by enzymatic purification using Ligase cleanup™ enzyme for 70 min. miRtagged mature miRNAs were then hybridized with the mouse miRNA V 1.5 panel (LBL-C0068-02) according to the instructions at nCounter® miRNA Expression Assay User Manual (NanoString Technologies, Seattle, WA, USA) for 22 h at 65°C. The unhybridized CodeSet was removed with automated purification performed on the nCounter Prep Station, and the remaining target probe complexes were transferred and bound to an imaging surface as previously described [28]. The data output was imported into nSolver™ 4.0 Analysis software (NanoString Technologies, Seattle, WA, USA). Raw data were exported as an Excel table for further bioinformatics analysis.

2.4. Bioinformatic Analysis

Volcano plots were created using SRPLOT tools based on the normalized counts to identify significant DE miRNAs. The R heatmap function (v1.0.12) was used to perform heatmap clustering. Three databases, miRWalk, TargetScan, and miRDB, were used to identify predicted miRNA target genes. The miRNA-target genes networks were plotted with Cytoscape software (which overlaps genes from the three aforementioned databases). Finally, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the predicted targets of the differentially expressed miRNAs in BC-M and BC-F groups were conducted using DIANA miRPath tools [29]. The STRING online tool was used to identify protein-protein interactions among identified key genes. STRING provides direct (physical) interactions and indirect (functional) associations that stem from computational prediction, knowledge transfers between organisms, and interactions aggregated from other (primary) databases. The miRNAs targeting *Bco1* mRNA were identified on the miRwalk platform using two filters: CDS and binding probabilities. A binding probability of 1 indicates higher quality prediction. Finally, experimentally validated microRNA-target interactions were assessed using miRTarbase.

2.5. Statistical Analysis

Comparisons between the vehicle-treated (control group, C) and β-carotene (BC)-treated groups were carried out for each sex (C-M/BC-M and C-F/BC-F). For biometric parameters, all data are shown as means and standard error of the mean (SEM). Statistical analyses were conducted using Graph Pad Prism 10 for Windows (GraphPad Software, La Jolla, CA, USA). Comparisons between groups were performed with one-way ANOVA with Uncorrected Fisher's LSD; differences were considered significant when $P< 0.05$. For miRNA expression profiling, the DESeq2 R package [30] was used to perform data normalization based on miRNA raw counts and the analysis of differentially expressed (DE) miRNA between sex-matched BC and C groups. miRNAs that showed > 0.5-fold change in BC-M vs. C-M and >1-fold change in BC-F vs. C-F with a P-value <0.05 were used as cut-offs to filter DE miRNAs. Similarly, a cut-off of $P< 0.05$ was used for significant enrichment results in GO and KEGG analyses. The top 10 enriched terms were plotted with dotplot using the clusterProfiler R package.

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3. Results

3.1. Biometric Parameters

Body weight, adipose tissue, and liver weights of recently weaned control and BC-supplemented male and female offspring born to mothers fed an obesogenic WD are shown in **Supplementary Table S1**. BC supplementation during the suckling period contributed to a significantly decreased body weight in male offspring compared to mice with obesogenic diet only (C-M= 13.73±0.53 g, BC-M= 12.45±0.17 g, P= 0.03), but not in female offspring (C-F= 11.85±0.37 g, BC-F= 11.13±0.35 g, P= 0.129). The BC-supplemented male offspring also displayed decreased retroperitoneal (C-M= 23.1±2.8 mg, BC-M= 18.2±0.8 mg, P= 0.027) and gonadal white adipose tissue mass (C-M= 129.2±15.8 mg, BC-M= 102.5±3.1 mg, P= 0.0309) compared with their sex-matched controls. No statistically significant changes in any biometric parameter were observed in female offspring.

3.2. Nanostring Analysis of miRNAs and Differentially Expressed miRNAs

The hepatic miRNAs affected by BC supplementation differed between male and female animals, with no common differentially expressed (DE) miRNAs found.

Figure 2 depicts DE miRNAs in the liver of male mice treated with BC compared to controls. The volcano plot (**Figure 22A**) shows that 10 miRNAs were differently expressed between the BC-M and the C-M groups. Specifically, one miRNA was downregulated, and nine were upregulated in the BC-M group. The clustered heatmap in **Figure 2B** shows the expression levels of these DE miRNAs in the two groups of male animals. Table 1 lists and summarizes the DE miRNAs in the BC-M vs. C-M group.

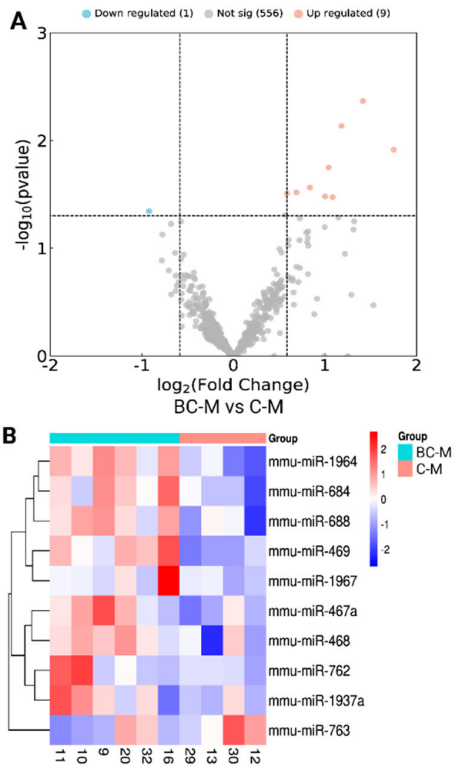

Figure 2. A) Volcano plot and **B)** Heatmap visualizing differentially expressed miRNAs in the liver of BC and Control male mice. In the volcano plot, the blue spots represent downregulated miRNAs

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and the pink upregulated miRNAs. Grey spots represent miRNAs that did not show significant changes between the two groups. C-M, Control-male; BC-M, β-carotene-male.

Table 1. Differentially expressed miRNAs in the liver of males with BC supplementation.

miRNA	Fold change	Regulation	*P*-value
mmu-miR-763	-1.893	Down	0.0453
mmu-miR-1937a/b	1.751	Up	0.0121
mmu-miR-762	1.41458	Up	0.004291
mmu-miR-468	1.181217	Up	0.007326
mmu-miR-1967	1.083272	Up	0.033513
mmu-miR-469	1.040029	Up	0.017799
mmu-miR-688	1.001268	Up	0.033073
mmu-miR-684	0.836395	Up	0.027278
mmu-miR-1964	0.687733	Up	0.030326
mmu-miR-467a	0.585563	Up	0.030996

Figure 3 shows results in females. There were 51 miRNAs differently expressed in the liver of the BC-F vs. C-F group. Of these, 42 were downregulated and 9 upregulated in the BC-supplemented females, as highlighted in the Volcano plot **(Figure 3A**). The clustered heatmap in **Figure 3B** shows the expression levels of these DE miRNAs in the two groups of female animals (note the heterogeneity within the C-F group)**. Table 2** lists the DE miRNAs in the BC-F vs. C-F group.

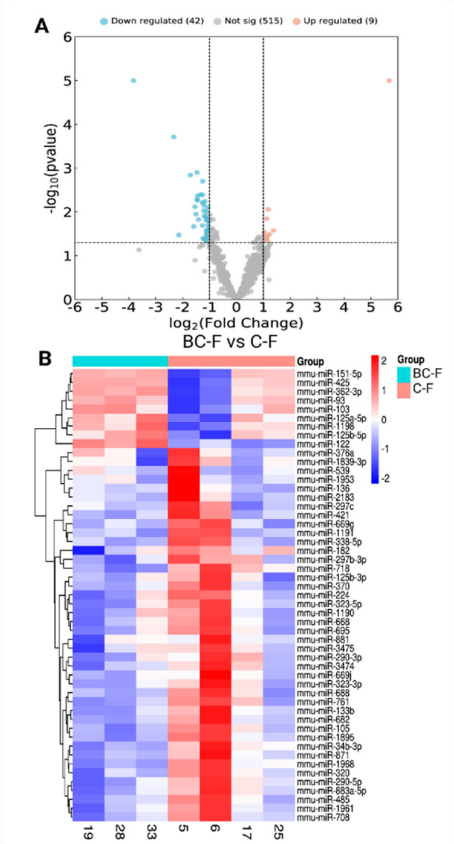

Figure 3. A) Volcano plot and **B)** Heatmap visualizing differentially expressed miRNAs in the liver of BC and Control female mice. In the Volcano plot, the blue spots represent downregulated miRNAs and the pink upregulated miRNAs. Grey spots represent miRNAs that did not show significant changes between the two groups. C-F, control-female; BC-F, β-carotene-female.

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Table 2. Differentially expressed miRNAs in the liver of females with BC supplementation.

miRNA	Fold change	Regulation	*P*- value
mmu-miR-1191	-14.1521	Down	3.09E-07
mmu-miR-2183	-5.01241	Down	0.000193
mmu-miR-376a	-4.40317	Down	0.033524
mmu-miR-1968	-3.26993	Down	0.001442
mmu-miR-539	-3.00592	Down	0.021402
mmu-miR-136	-2.90541	Down	0.007614
mmu-miR-669g	-2.8221	Down	0.011202
mmu-miR-338-5p	-2.76262	Down	0.001253
mmu-miR-682	-2.75437	Down	0.005321
mmu-miR-323-5p	-2.72259	Down	0.005302
mmu-miR-290-5p	-2.7219	Down	0.004294
mmu-miR-182	-2.65349	Down	0.014895
mmu-miR-320	-2.50812	Down	0.004008
mmu-miR-761	-2.40692	Down	0.00612
mmu-miR-871	-2.40115	Down	0.004071
mmu-miR-1839-3p	-2.40041	Down	0.020481
mmu-miR-34b-3p	-2.38487	Down	0.001983
mmu-miR-881	-2.36257	Down	0.040452
mmu-miR-224	-2.32138	Down	0.009298
mmu-miR-1961	-2.31858	Down	0.007012
mmu-miR-125b-3p	-2.2945	Down	0.041596
mmu-miR-297c	-2.28392	Down	0.012285
mmu-miR-421	-2.25396	Down	0.005722
mmu-miR-883a-5p	-2.21618	Down	0.013146
mmu-miR-3474	-2.19501	Down	0.04617
mmu-miR-3475	-2.17952	Down	0.033841
mmu-miR-297b-3p	-2.16784	Down	0.015613
mmu-miR-1953	-2.16066	Down	0.025989
mmu-miR-695	-2.1502	Down	0.039103
mmu-miR-323-3p	-2.13416	Down	0.028574
mmu-miR-133b	-2.13306	Down	0.010116
mmu-miR-370	-2.12883	Down	0.03029
mmu-miR-1895	-2.08625	Down	0.007972
mmu-miR-688	-2.07886	Down	0.047323
mmu-miR-485	-2.0763	Down	0.014138
mmu-miR-290-3p	-2.07055	Down	0.020309
mmu-miR-105	-2.06476	Down	0.015803
mmu-miR-669j	-2.06456	Down	0.035964
mmu-miR-1190	-2.0396	Down	0.048281
mmu-miR-708	-2.03612	Down	0.016461
mmu-miR-668	-2.03022	Down	0.029321
mmu-miR-718	-2.01753	Down	0.024074
mmu-miR-122	51.125	Up	1.74E-25
mmu-miR-103	2.598296	Up	0.026379
mmu-miR-125b-5p	2.352268	Up	0.032295
mmu-miR-362-3p	2.265612	Up	0.008745
mmu-miR-151-5p	2.229765	Up	0.035369
mmu-miR-125a-5p	2.19763	Up	0.044985

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mmu-miR-1198	2.186721	Up	0.014346
mmu-miR-93	2.170918	Up	0.041012
mmu-miR-425	2.050926	Up	0.029188

3.3. Gene Ontology (GO) Analysis of the Differentially Expressed miRNAs Target Genes

The biological significance of the DE miRNAs integrated signature was approached via their target genes. To this end, predicted targets of the down and upregulated miRNAs in the liver of BC-supplemented male and female offspring were identified (see section 3.4), and GO enrichment analyses were performed on the respective whole gene sets. GO categories were assigned to the target genes according to the Biological Processes (BP) in which the gene products are involved, the Cellular Components (CC) to which the gene products are located, and their Molecular Function (MF).

Figure 4 shows the top 10 significant results of each of the three GO categories for the predicted targets of downregulated (**Figures 4A-C**) and upregulated (**Figures 4D–F**) DE miRNAs in the liver of BC male mice. Target genes of the downregulated miRNAs in males showed enrichment in BPs such as the ERK1 and ERK2 cascade and its regulation (**Figure 4A**); CCs related to synapses (**Figure 4B**); and MFs such as peptide binding and phosphatidylinositol 3-kinase regulatory subunit binding (**Figure 4C**). Meanwhile, target genes of the upregulated miRNAs in males showed enrichment in BPs related to neural developmental and organization processes and regulation of circadian rhythm (**Figure 4D**); CCs related again to synapses (**Figure 4E**); and MFs such as protein serine/threonine kinase activity and GTPase activator and regulator activities (**Figure 4F**).

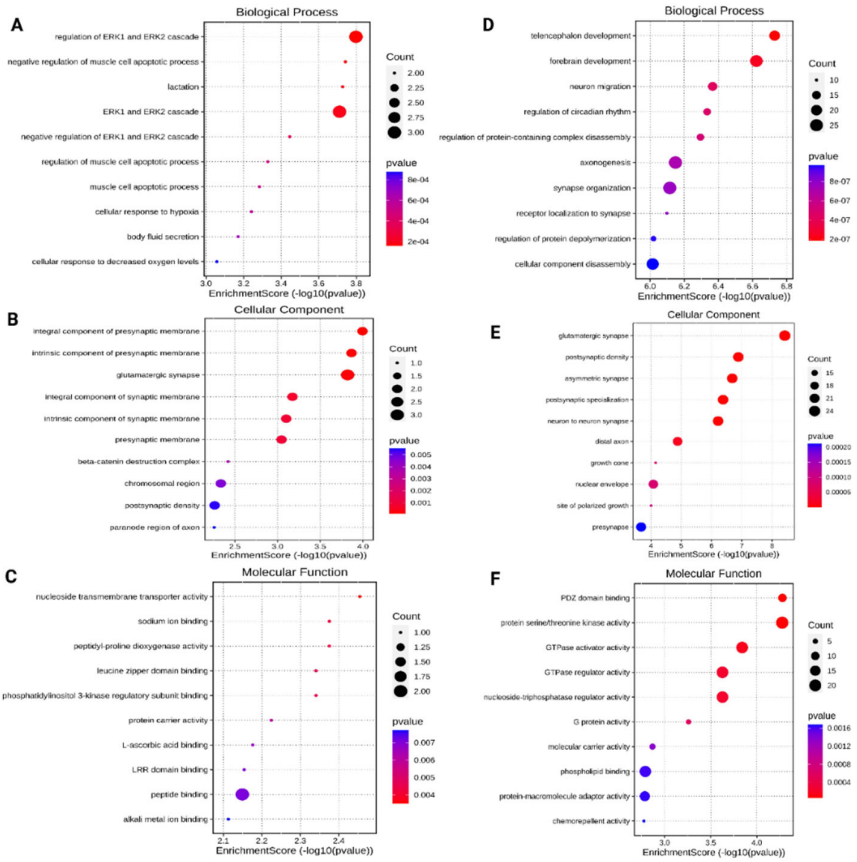

Figure 4. Gene Ontology analysis of target genes of miRNAs differentially expressed (DE) in the liver of BC-treated male offspring. Panels A to C (left) correspond to the downregulated miRNA targets, and panels D to F (right) to the upregulated miRNAs targets. The panels show the top 10 most represented Biological Processes (A, D), Cellular Components (B, E), and Molecular Functions (C, F) among the DE miRNAs targets. The colour of each dot represents the P value of each term involved

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in the analysis. The size of each dot represents the counts of overlapped genes between the input genes and the total gene list on GO pathway.

In females, predicted targets of the miRNAs downregulated in the liver with BC treatment associated with BPs such as cellular glucuronidation and metabolic processes (**Figure 5A**) and with diverse CCs and MFs, as indicated in **Figure 5B** and **5C**, respectively. Finally, targets of the upregulated miRNAs in the liver of BC females showed enrichment in processes related to the regulation of developmental growth and cell size as BPs (**Figure 5D**); transcription regulator complex as CC (**Figure 5E**); and nuclear receptor activity, ligand-activated transcription factor activity, and protein serine/threonine kinase activity as MFs (**Figure 5F)**, among others.

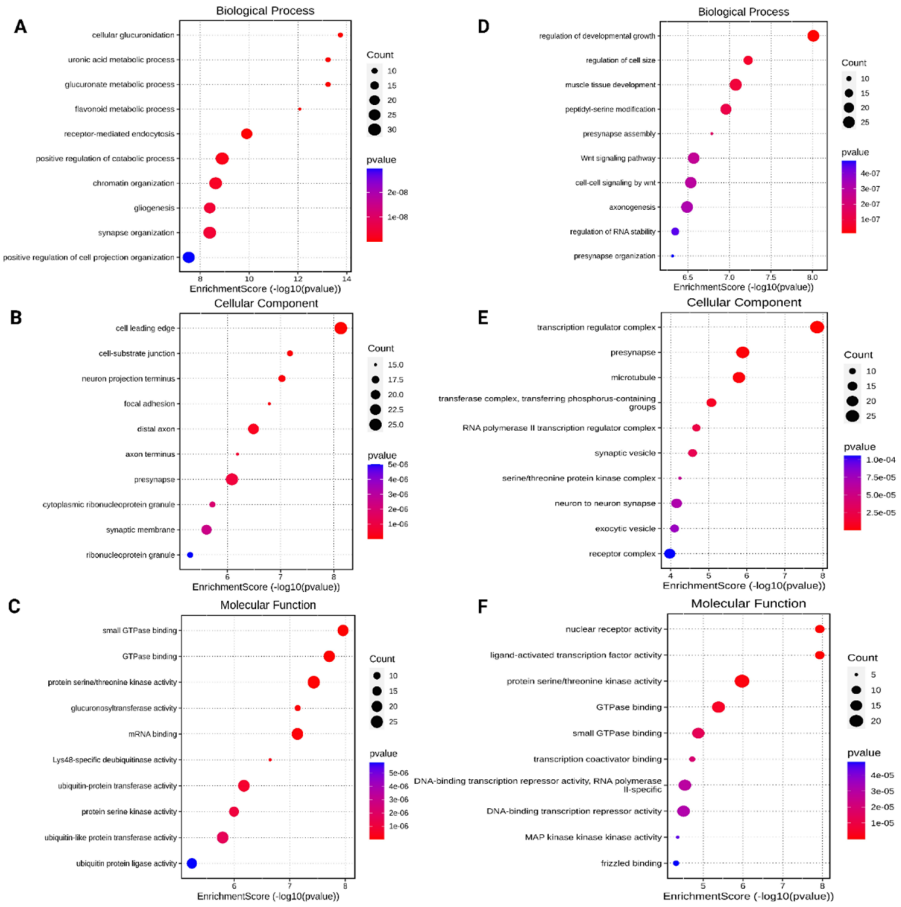

Figure 5. Gene Ontology analysis of target genes of miRNAs differentially expressed (DE) in the liver of BC-treated female offspring. Panels A to C (left) correspond to the downregulated miRNAs targets, and panels D to F (right) to the upregulated miRNAs targets. The panels show the top 10 most represented Biological Processes (A, D), Cellular Components (B, E), and Molecular Functions (C, F) among the DE miRNAs targets. The colour of each dot represents the P value of each term involved in the analysis. The size of each dot represents the counts of overlapped genes between the input genes and the total gene list on GO pathway.

3.4. KEGG Pathway Functional Enrichment Analysis

Figure 6 shows the top KEGG-enriched pathways associated with predicted targets of the DE miRNAs in the liver of BC-supplemented offspring of each sex. Fifty-two pathways were significantly enriched in the BC males (p-value <0.05). Targets of the downregulated miRNA associated significantly with four KEGG pathways: Amyotrophic lateral sclerosis, Alzheimer's disease, Pathways of neurodegeneration, and Hedgehog signaling (**Figure 6A**). The 10 most significantly enriched KEGG pathways of targets of the upregulated miRNAs in BC-M included Cell adhesion

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molecules, Hedgehog signaling, ErbB signaling, and Insulin resistance, among others (**Figure 6B**). In the BC females, 130 KEGG pathways were significantly enriched. Of them, the top 10 enriched among targets of the downregulated miRNAs in BC-F included Ascorbate and aldarate metabolism, Porphyrin metabolism, Pentose and glucuronate interconversions, Chemical carcinogenesis, Bile secretion, and Drug metabolism, among others (**Figure 6C**); additionally, Steroid hormone biosynthesis and Retinol metabolism were significantly enriched (p-value <0.05, data not shown). Finally, the top 10 significantly enriched pathways among targets of the upregulated miRNAs in BC-F included among others Notch signaling, Cushing syndrome, Circadian rhythm, Non-small/small cell lung cancer, Breast cancer, Hedgehog signaling, and Endocrine resistance (**Figure 6D**).

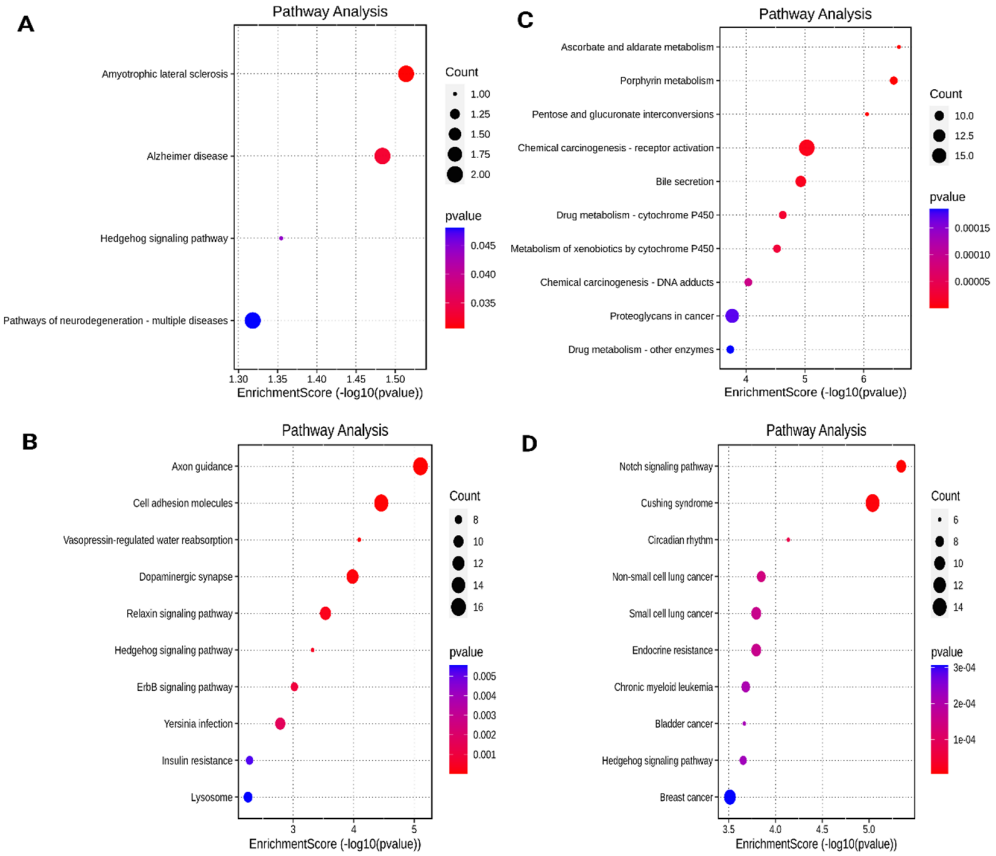

Figure 6. KEGG analysis of target genes of downregulated (A) and upregulated (B) miRNAs in the liver of BC- supplemented males, and of downregulated (C) and upregulated (D) miRNAs in the liver of BC-supplemented females. The colour of each dot represents the P value of each term involved in the analysis. The size of each dot represents the counts of overlapped genes between the input genes and the total gene list on KEGG pathway.

3.5. Construction of miRNA-Gene Interactional Networks and Identification of miRNAs with Bco1 as Target

Based on the miRNA-gene prediction pairs, miRNA-target gene networks were plotted with Cytoscape software (Supplementary Material **Figure S1)**. Target genes of the 61 DE miRNAs in the liver of BC-supplemented offspring were predicted by three tools: miRWalk database, TargetsScan, and miRDB. A total of 1504 predicted target genes were obtained, 543 in male mice and 961 in female mice. In both sexes, the miRNA-gene networks revealed several genes targeted by multiple DE miRNAs. In males, miR-763, the only downregulated miRNA, was predicted to target a low number of genes, while miR-762 had the highest number of predicted targets, followed by miR-1967, miR-467a-5p, miR-684, miR-468-3p, miR-688, and miR-1964-3p. In females, among the downregulated miRNAs, miR-182-5p had the highest number of targets, followed by miR-320-3p, miR-133b-3p, miR-370-3p, and miR-485-5p; whereas among the upregulated miRNAs, miR-93-5p had the highest

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predicted number of targets, followed by miR-125a-5p, miR-125b-5p, miR-103-3p, miR-122-5p, miR-362-3p, miR-425-5p, and miR-151-5p. The Supplementary Material shows the whole networks (**Figure S1)**.

In an attempt to decipher the major functions of the miRNAs DE in the liver of BC-supplemented offspring, we further focused on predicted targets overlapping in both sexes. To be noted, even if no DE miRNAs in common were found in the liver of males and females supplemented with BC, it is possible that the target genes of different DE miRNAs partially overlap since the same mRNA can be regulated by different miRNAs [31]. Furthermore, the co-regulatory miRNAs might respond differently to treatments/interventions, so some are upregulated and others downregulated. A Venn diagram (**Figure 7**) showed 456 uniquely predicted target genes of DE miRNAs in the male mice, 814 uniquely predicted target genes in the female mice, and a total of 86 predicted target genes overlapping in the two sexes: 39 overlapping targets of upregulated miRNAs in either sex, 38 overlapping targets of downregulated miRNAs in females and upregulated miRNAs in males, and 9 overlapping targets of upregulated miRNAs in males, and down and upregulated miRNAs in females. Twenty-two DE miRNAs - six in males (mmu-miR-762, -684, -467, -1967, -468, -1964) and 16 in females (mmu-miR-182-5p, -5p, -320-3p, -133b-3p, -485-5p, -668-3p, -224-5p, -323-3p, -708-5p, -1968-5p, -103-3p, -125a-5p, -93-5p, -125b-5p, -362-3p, -122-5p) - were predicted to be involved in the targeting of these 86 overlapping genes. The overlapping genes and their potential regulatory DE miRNAs in the liver of male and female BC-supplemented mice are listed in Supplementary **Table S2.** Out of the 86 genes that overlapped, 47 were predicted targets of miRNAs that were regulated in opposite directions (up or down) between sexes. KEGG pathway adscriptions of the 86 overlapping targets in both sexes are presented in **Table S3**. It is worth noting that *Pik3r1* was the most involved gene in multiple KEGG pathways (see Supplementary Material **Table S3**).

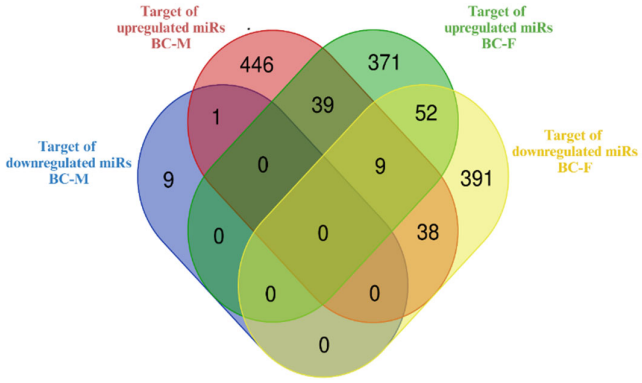

Figure 7. Venn diagram of predicted target genes of the miRNAs differentially expressed (DE) in the liver of BC-supplemented males (BC-M) and females (BC-F). Created with Bioinformatics & Evolutionary Genomics.

miRNA-target gene mRNA networks were constructed through Cytoscape considering the 86 overlapping genes and the 22 DE miRNAs potentially involved in their regulation (**Figure 8**). In the BC males, 98 pairs of upregulated miRNAs-downregulated mRNAs were predicted (**Figure 8A**). Some genes (*Cux1, Blcap, Zc3h12c, Lhx6, Nfib,* and *Cadm2*) paired with more than one of the upregulated miRs in BC-M liver, acting at intersections between miRNAs (**Figure 8A**). In the BC females, 109 miRNAs-gene pairs were predicted, of which 51 pairs involving miRNAs downregulated (**Figure 8B**) and 58 pairs involving miRNAs upregulated (**Figure 8C**). There were 5 target genes at the intersections among downregulated miRNAs (*Ubn2, Cux1, Otud7b, Prlr,* and *St8sia2*), and 7 target genes at the intersections among upregulated miRNAs (*Etv3, Atxn1, Trp53inp1, Rora, Snob1, Cacna1b,* and *Kcnk10*). Out of the DE miRNA-gene pairs predicted in male animals, mmu-miR-684, -1967, and -762 are experimentally validated regulatory miRNAs for *Lhx6*, *Nav1*, and *Xpo7*, respectively, whereas out of the pairs predicted in female animals, mmu-miR-125b-5p, -362-3p, -122-

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5p, and -103-3p are experimentally validated regulatory miRNAs for *Abtb1*, *Atxn1*, *Slc7a1*, and *Pik3r1*, respectively (source miRTarbase).

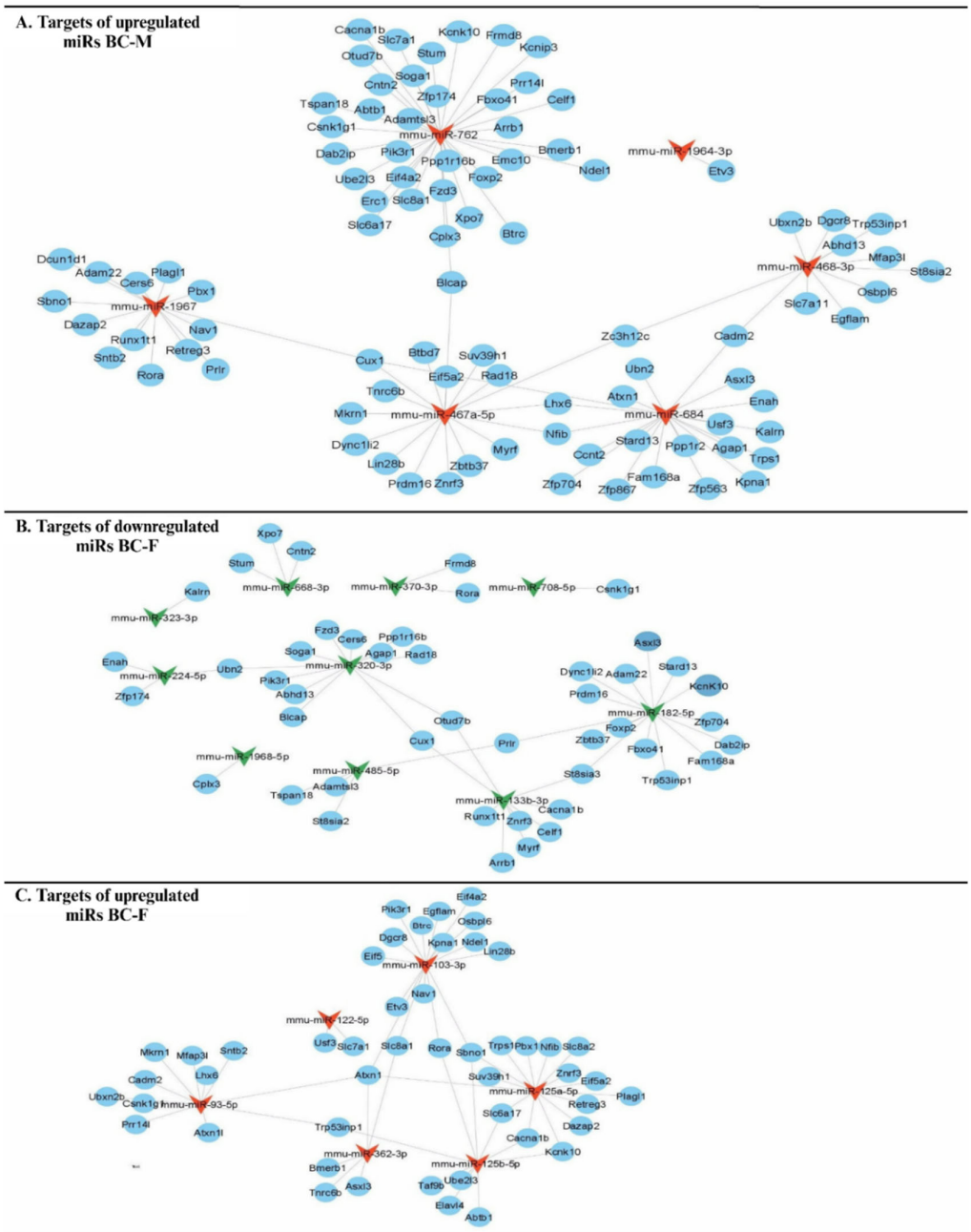

Figure 8. miRNA-gene interaction networks of the 86 target genes overlapping in both sexes. **A)** Targets of upregulated miRs in males. **B**) Targets of downregulated and **C**) upregulated miRs in females.

Besides the systematic bioinformatic approach, a targeted approach was used for *Bco1*, since this gene encodes the crucial enzyme (beta-carotene-15, 15'-oxygenase) for the cleavage and metabolism of BC in mammals [32]. Predicted miRNAs regulating the *Bco1* gene were searched on the miRwalk platform. A total of 990 miRNAs were found. Of them, focusing on the results of our microarray, six DE miRNAs were predicted to target the *Bco1* transcript, namely mmu-miR-763 in the male offspring and mmu-miR-668, -105, -370, -323-3p, and -290-3p in the female offspring (**Table 3**). All the DE

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miRNAs with *Bco1* as a potential target were downregulated in the liver of offspring born to WD-fed dams following BC supplementation during suckling.

Table 3. DE miRNAs in the liver of BC-supplemented offspring potentially regulating *Bco1*.

Gene	Sex	miRNAs downregulated	miRNAs upregulated
Bco1	Male	mmu-miR-763	-
	Female	mmu-miR-668, -105, -370, -323, -3p, -290-3p	-

3.6. Protein-Protein Interaction Network Analysis

To better understand the interactions among predicted targets of the miRNAs regulated by BC in the liver of young mice born to WD-fed mothers, protein-protein interaction (PPI) networks were constructed using the STRING database. Shown in **Table 4** are the top 10 hub genes among the 86 overlapping genes potentially regulated by BC supplementation in both sexes, ranked according to their node degree. The higher the node degree, the higher the core level of the gene in the network. The whole networks are shown in the Supplementary Material (**Figure S2**)

Table 4. Top 10 genes in the protein-protein interaction networks involving the DE miRNAs target genes overlapping across sexes, ranked by node degree.

Males upregulated		Females downregulated		Females upregulated	
Genes	**Node Degree**	**Genes**	**Node Degree**	**Genes**	**Node Degree**
Pik3r1	23	*Pik3r1*	29	*Pik3r1*	23
Arrb1	16	*Arrb1*	16	*Atxn1*	19
Kalrn	15	*Xpo7*	16	*Btrc*	13
Atxn1	13	*Kalrn*	13	*Suv39h1*	12
Cacna1b	9	*Cacna1b*	11	*Eif4a2*	10
Ube2l3	9	*Celf1*	11	*Erc1*	9
Xpo7	9	*Foxp2*	11	*Rora*	8
Btrc	8	*Cux1*	9	*Ube2l3*	7
Dab2ip	8	*Adam22*	6	*Cacna1b*	6
Ndel1	8	*Cntn2*	6	*Dgcr8*	6

4. Discussion

BC is a natural pigment with nutritional functions and interesting bioactive properties in adult animals, including hepatoprotective effects. Maternal unbalanced diets are known to cause malprogramming and affect miRNA expression in the liver of offspring. In the present study, we aimed to determine the impact of BC supplementation during the suckling period on the microRNA profile in the liver of young mice exposed prenatally and during lactation to an obesogenic maternal diet. We studied recently weaned offspring of both sexes and used hybridization to microarrays for miRNA profiling. Identifying sex-dependent responses is crucial to determining new targets, personalizing nutrition and treatment methods, and allowing translation to human health. Although RNA sequencing is increasingly been used to perform comprehensive analyses of miRNA expression profiles, it has been demonstrated that next-generation sequencing and microarray measurements give similar results [33].

BC supplementation differentially affected the hepatic miRNA expression profile in male and female animals, with no overlap in DE miRNAs between sexes and a stronger impact in the females. A smaller number of miRNAs responded to BC supplementation in males. Additionally, with the

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notable exception of miR-122 in females (discussed below), most DE miRNAs changed within a small range in both sexes. The low fold-change observed may be due to upregulated mmu-miR-468-3p in males and mmu-miR-103-3p in females. In the present study, these miRs were identified in silico as negative regulators of *Dcgr8* (encoding DROSHA), and the efficiency of miRNA processing by DROSHA crucially determines the abundance of miRNAs [34].

The liver is an organ with recognized sexually dimorphic metabolic and cellular responses and is extremely sensitive to the action of sexual hormones [35,36]. Further, the effects of dietary BC on gene expression are also highly organ and sex-specific [37]. Our results fit in this perspective since changes in the hepatic miRNA profile with neonatal BC strongly differed between sexes. Particularly noteworthy was the sex-dependent response to early life BC supplementation of hepatic miR-122 expression, markedly and significantly upregulated (x51-fold) in the female offspring and unaffected in the male offspring. Of note, miR-122 has been described as a female-predominant miRNA related to nutritional conditions (induced upon starvation) in rats [38]. miR-122 is the predominant liver miRNA, making up 70% of the total miRNA population [39]. miR-122 is a critical regulator of hepatic homeostasis and lipid metabolism, and its activity decreases lipogenesis and suppresses the pathogenic progress of hepatocellular carcinoma [40]. Increased liver miR-122 expression levels with neonatal BC, as observed in the female offspring, could thus represent a beneficial effect. Further, miR-122 expression is controlled by miR-370 [41] and, interestingly, a maternal high-fat diet was found to decrease miR-122 expression and increase miR-370 expression in the liver of recently weaned mouse offspring [19], changes opposite to those observed here in the female offspring of high-fat/high-sucrose (WD)-fed dams following BC supplementation, namely increased miR-122 and decreased miR-370 expression (Table 2). miR-370 directly down-regulates CPT1α, the rate-limiting enzyme in fatty acid β-oxidation [41].

We approached the potential functions of the DE miRNAs in the liver of BC males and females by GO annotation and KEGG enrichment analysis of predicted targets and the construction of miRNA-gene interaction networks. In male animals, significant GO terms retrieved pointed to specific biological processes potentially relevant to maintaining homeostasis in the liver, such as the ERK1/ERK2 cascade [42]. At the same time, KEGG analysis of targets of DE miRNAs in the liver of BC males showed enrichment for genes in the Hedgehog signaling pathway involved in regulating hepatic lipid metabolism and its zonation [43]. Interestingly, enriched pathways in the BC males also included neurodegenerations/Alzheimer's disease and insulin resistance, processes that are related to each other [44] and potentially to BC nutrition and metabolism. A connection between neurodegenerations/ Alzheimer's disease and BC is indicated by results showing modulation of dietary BC metabolism by the APOE genotype: following a BC diet, engineered mice expressing the human APOE4 isoform had lower plasma and tissue BC levels due to higher hepatic (but not intestinal) *Bco1* expression compared with mice expressing human APOE3 [45]. The APOE4 genotype is the strongest genetic risk factor for Alzheimer's disease [46]. The connection is also suggested by research pointing to the protective effects of BC on cognitive function and specifically against Alzheimer's disease [5,6]. A connection between insulin resistance and BC may involve effects on the low-density lipoprotein (LDL) fraction. LDL is the main carrier of circulating BC, transporting about 60%–70% of total serum BC [47], and there is evidence that BC can inhibit the oxidative modification of LDL [48]. High oxidized LDL levels are associated with insulin resistance [49], metabolic syndrome [50], and atherosclerosis [51]. Oxidized LDL is also neurotoxic and has been related to Alzheimer's disease [52], even if this latter condition has multifactorial etiology. Serum BC levels in humans are inversely associated with insulin resistance [53] and metabolic syndrome [54] and are reduced in patients with Alzheimer's disease [55,56].

As in males, Hedgehog signaling was identified as one of the top enriched KEGG pathways of targets of DE miRNA in the liver of female offspring supplemented with BC. However, neurodegenerations/Alzheimer's disease and insulin resistance were not. Instead, enrichment in targets related to drug metabolism and cancer was revealed for DE miRNAs in the liver of BC-supplemented females. It is well established that similar to humans, male mice have lower insulin sensitivity than female mice and are more susceptible to unhealthy diet-induced metabolic disorders

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[57–59]. Therefore, epigenetic modulation of these health endpoints by neonatal BC supplementation (for instance, through effects on miRNAs in the liver and possibly other tissues) could be particularly interesting in males. Overall, supplementing BC during the suckling period impacts the hepatic expression of miRNAs in recently weaned mice born to mothers fed an unbalanced diet, and GO and KEGG analysis suggest that the affected miRNAs target genes involved in clinically relevant pathways modulated in a beneficial manner by BC/vitamin A in the adult animals. Therefore, changes in these miRNAs could represent early epigenetic mediators of long-term effects of early postnatal BC supplementation in offspring exposed to an unbalanced maternal diet during gestation and lactation.

Six DE miRNAs in males and 16 DE miRNAs in females were predicted through *in silico* analyses to regulate 86 overlapping genes across both sexes. Forty-seven of these overlapping genes (55% percent) were targeted by miRNAs with opposite (down or up) regulation between sexes. Nutritional or functional associations to BC or BC derivates are described in the literature for some of the overlapping genes, such as *Cntn2* [60], *Etv3* [61], *Foxp2* [62], *Otud7b* [63], and *Pbxl* [64]. Additionally, among the 47 predicted overlapping targets of miRNAs affected in opposite senses across sexes was the *Rora* gene encoding the retinoic acid receptor-related orphan receptor alpha transcription factor. Interestingly, sex-dependent differences in the expression of the *Rora* gene in the liver during aging have been described [65]. Further, the expression of miR-122 – a miRNA with a marked sex-dependent response in our experiment- is induced in liver cells by saturated free fatty acids in a RORα-dependent manner [66]. *Rora* is a circadian rhythm gene [67], an enriched pathway in our GO analysis for males and KEGG analysis for females.

Bioinformatics analysis of interactions between predicted target gene products of miRNAs differentially expressed upon BC supplementation revealed many connections involving *Pik3r1*. This gene was a predicted target of DE miR-762 in males and of miR-320-3p and miR-103-3p in females and is a validated target of miR-103-3p (Table S2). *Pik3r1* encodes a regulatory subunit of phosphoinositide 3-kinases (PI3K). PI3K are crucial components of the PI3K/AKT/mTOR pathway regulating cell growth, metabolism, survival, apoptosis, and autophagy [68]. The *Pik3r1* product binds, stabilizes, and inhibits the PI3K catalytic subunit, thus exerting a complex regulatory role. It is involved in modulating the metabolic actions of insulin, and its mutation has been associated with insulin resistance [69]. The *Pik3r1* protein product also has tumor suppressor potential since its reduced levels favor the constitutive activation of downstream Akt signaling, which can induce carcinogenesis [70]. Previous studies showed BC can activate the PI3K/AKT/mTOR signaling pathway to attenuate apoptosis and autophagy induced by LPS in intestinal epithelial cells [71] and glycation end products in cardiomyocytes [72]. Though these examples are not in liver cells, results herein in this work suggest the possible involvement of BC effects on miRNAs targeting *Pik3r1*.

Interestingly, both in male and female offspring, DE miRNAs were found targeting *Bco1*, and all of them were downregulated, suggesting the possible upregulation of hepatic *Bco1* expression following oral BC supplementation irrespective of sex. Dietary BC conversion to vitamin A retinoids through BCO1 decreases adiposity [73,74], circulating cholesterol [75], and atherosclerosis progression in various experimental animal models [76,77]. In humans, responses to dietary BC may be widely variable due to genetic variants in the BCO1 coding gene [78,79]. Additionally, there is emerging evidence that BCO1 may have functional roles in regulating metabolism and cellular fate independent of its role in carotenoid cleavage, and these roles seem to involve the regulation of miRNAs [12,80].

This is the first report to evaluate the impact of neonatal BC supplementation in the context of an obesogenic maternal diet on the miRNA profile in the liver of offspring. Our study provides a global vision that BC supplementation in early life can impact tissue miRNA expression in a sex-dependent manner and that sexual differences in metabolic programming could rely on miRNAs, among other factors. The present study has some limitations that should be noted. First, our results are based on a mouse model with a small sample size and are limited to the short term. An in-depth investigation should be performed to ascertain the long-term programming impact of observed effects in recently weaned animals and the translatability of findings to humans. Second, we

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performed a bioinformatics analysis based on microarray data without RNA expression validations by real-time qPCR; thorough validation and further functional experiments should be performed in the future to investigate specific candidate miRNAs and their downstream functions directly. Given the complexity of the biological response predicted, further exploration is required to uncover interesting aspects of gene regulation by miRNAs affected by BC supplementation in early life.

To sum up, findings in this work identify distinct miRNAs differentially expressed with BC supplementation in the female and male liver of young mice born to WD-fed mothers and provide an overall vision of their potential functional impact. The current study and integrated analysis may open new avenues for research toward understanding the molecular mechanisms associated with the programming effects of BC supplementation early on in offspring exposed to a maternal unbalanced (Western) diet. Further studies are required to decipher the long-term impact on liver metabolism and health and the underlying molecular mechanisms of these miRNAs.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, **Table S1:** Biometric parameters at sacrifice; **Table S2:** Overlapping target genes in both sexes of miRNAs differentially expressed with neonatal beta-carotene supplementation; **Table S3:** KEGG pathway adscriptions of overlapping target genes in both sexes of miRNAs differentially expressed with neonatal beta-carotene supplementation; **Figure S1:** miRNA-gene interaction networks; **Figure S2:** Protein-protein interaction (PPI) networks of predicted targets of the miRNAs differentially expressed with neonatal beta-carotene supplementation.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors do not have any conflict of interest to disclose.

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